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Review

Functional nanomaterials and nanoprobes for amplified biosensing☆

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ABSTRACT

The introduction of functional nanomaterials into biosensing fields advanced analytical chemistry and opened a series of concepts for design of new analytical and tracing tools. It not only improved the stability and biocompatibility of biosensing interfaces, also has greatly enhanced the biosensing response to recognition event, thus met the need for detection of trace amounts of a wide variety of analytes and acquisition of ultraweak biological signals, and promoted the development of life science, biomedicine, food and environmental science. Different nanomaterials have been functionalized to modify the biosensing interfaces for accelerating the electron transfer, lowing the activation energy, and/or concentrating the target analytes, or to act as nanoprobes for labeling the recognition molecules, in which the nanomaterials can act as catalysts, enzyme mimics, signal emitters or carriers for loading of signal molecules to produce amplified signals. By coupling with the specificity of biological recognition events, the functional nanomaterials and nanoprobes have been quickly developed for highly sensitive and specific biosensing both in vitro and in vivo. This review focuses on the significant advances in use of functional nanomaterials for design of amplified biosensing strategies. The main works in this group are also summarized to expound the views and research thinking in the amplified biosensing.

Contents

☆ Published in honor of the 70th birthday of Professor Joseph Wang.

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1. Introduction

The unique properties of nanoscale materials (1–200 nm) offer excellent platforms for electronic or optical signal transduction and design of a new generation of bioelectronic and biosensing devices. To solve the drawbacks of nanomaterials in biocompatibility and biological recognition ability, many efforts have been made on their biofunctionalization with a wide variety of biomolecules, small organic molecules, polymers or even entire living cells via noncovalent or covalent interaction. These functionalized nanomaterials as well as some biocompatible nanoparticles can be directly used to modify the biosensing interfaces for accelerating the electron transfer, lowing the activation energy as enzyme mimics or catalysts, and/or concentrating the target analytes, to carry the signal molecules such as enzymes, redox reagents, organic dyes, fluorescent proteins and quantum dots (QDs), or to act as electronic or optical signal emitters. These functions greatly enhance the response of electronic, optical, and photoelectrochemical biosensing to recognition event, and improve the analytical performance of biosensing interfaces. Therefore, the introduction of functional nanomaterials into biosensing fields essentially solved the limitation of the classical analytical methodologies in amplifying the signal via traditional physical methods or simple chemical and biocatalytic processes to meet the need in detection of trace amounts of a wide variety of analytes and acquisition of ultraweak biological signals, which advances analytical chemistry and opens a series of concepts for design of new analytical and tracing tools. These achievements also promote the development of life science, biomedicine, food and environmental science [1].

Besides the enhanced biosensing response, the excellent selectivity or specificity can also be achieved by functionalization of nanomaterials with biomolecules, including DNA, aptamers, proteins, antibody/antigens, lectins, peptides, as specific recognition or/and signal triggering element. Thus the functional nanomaterials can be endowed with the abilities of both recognitions to target molecules and signal amplification. They have been extensively employed for excellent signal transduction of biological phenomena in development of sensitive biosensing strategies [2]. By introducing the functionalized nanomaterials with the amplification technologies based on DNA recognition or molecular biological protocols, such as rolling circle amplification (RCA) [3], target-induced repeated primer extension [4], hybridization chain reaction [5], ligase chain reaction [6], target DNA recycling amplification [7] with polymerase [8], exonuclease [9] and endonuclease [10], circular strand-replacement polymerization [11] and assistant DNA recycling [12], the detection sensitivity has been further improved. For example, with electrochemical stripping analysis a dual signal amplification strategy by RCA and quantum dots tagging can quantitatively detect protein down to 16 molecules in a 100-μL sample [13] and another ternary signal amplification strategy by target DNA recycling, RCA and quantum dots tagging can detect DNA down to 11 aM with a linear range of 6 orders of magnitude [14]. The high sensitivity makes it possible to detect undetectable targets by traditional methods, such as some disease markers, biological threat agents and pathogens [15].

In view to the breakthrough in detection sensitivity and specificity, and easy miniaturization of related detection systems, functional nanomaterials and nanoprobes have been quickly developed for highly sensitive and specific biosensing both in vitro and in vivo, especially the in situ analysis of intracellular functional biomolecules, by coupling with electrochemical detections such as voltammetric analysis, impedance analysis, capacitance analysis, electrochemiluminescent analysis and photoelectrochemical analysis; optical detections such as chemiluminescent analysis, fluorescent analysis and infrared, ultraviolet and Raman analysis; mass spectrometric analysis; imaging or visualization technologies such as grayscale scanning imaging, scanning electrochemical microscopic imaging, chemiluminescence imaging, fluorescence imaging, Raman spectral imaging and mass spectral imaging. The established methods can conveniently be used in the detections of small biomolecules, proteins [16], DNA, mRNA, microRNA [17], cells, cell surface glycans and intracellular functional biomolecules. Furthermore, the detection channels and throughput can be greatly improved by coupling with array techniques. This review focuses on the significant advances in use of functional nanomaterials for design of amplified biosensing strategies. The main works in this group are also summarized to expound the views and research thinking in the amplified biosensing.

2. Functional nanomaterials for amplified electrochemical biosensing

2.1. Direct electron transfer for amplified electrochemical biosensing

The use of gold nanoparticles with excellent biocompatibility for the creation of electrochemical sensing devices is an extremely promising prospect [18]. The possibility for direct electron transfer between conductive gold nanoparticles and redox cytochrome c [19] and horseradish peroxidase (HRP) [20] paved the way for the construction of amperometric biosensors. The small size of gold nanoparticles and the nanoscale contours allows them to come into a close proximity of the active center of the enzyme, which lowers the electron-transfer distance between the electrode and the redox site embedded in the protein and thus accelerates the electron transfer. Moreover, the Au nanoparticles can act as nanoscale electrodes to electrically communicate between redox proteins and bulk electrode materials [21]. Thus, Xiao et al. [21] assembled a spatially ordered monolayer of gold nanoparticles to immobilize HRP on electrode surface to develop for the first time a sensitive electrochemical biosensor for hydrogen peroxide. Owing to the presence of long nonconductive spacer, the biosensing needed a diffusional electron-transfer mediator (catechol). In order to decrease the nonconductive spacer between gold electrode surface and gold colloid nanoparticles, this group then assembled gold nanoparticles and HRP on the electrode surface with a short-chained monolayer, which showed the direct electrochemistry of HRP for detection of hydrogen peroxide [22]. At the same time, a series of electrochemical sensors were built by the electrostatic crosslinking of gold nanoparticles with electron-transfer mediator such as bipyridinium cyclophanes as well as their complex [23,24]. By mixing gold colloid in carbon paste to prepare gold colloid nanoparticle modified carbon paste electrodes, Ju et al. [25] presented a method to immobilize protein on the electrode surface and study the direct electron transfer and biosensing of proteins such as cytochrome c, tyrosinase [26], myoglobin [27] and hemoglobin [28]. This method combines the advantageous features of colloidal gold nanoparticle and carbon paste technology. The direct electron transfer of glucose oxidase (GOD), with regard to the GOD(FAD) to GOD(FADH2) conversion, could be facilitated, which led to a reagentless biosensor for amplified glucose detection [29]. The redox potential of protein adsorbed on the electrode surface was close to that of native state in solution, suggesting that most molecules preserved their native structure after the adsorption process due to the presence of the gold nanoparticles.

Due to the good hydrophilicity and biocompatibility, mesoporous silica [30], zeolite [31], zironium dioxide nanoparticles [31], mesocellular silica-carbon nanocomposite foam (MSCF) [32], PdNPs doped MSCF nanocages [33] were also used to accelerate the electron transfer of redox proteins for biosensing. The hemoglobin immobilized in the mesopores of hexagonal mesoporous silica

(HMS) showed the direct electron transfer with two couples of redox peaks corresponding to the adsorbed and the intercalated hemoglobin, respectively [34]. Li's group [35] reported the electron transfer of hemoglobin in bimodal mesoporous silica (BMS) and chitosan inorganic–organic hybrid film. The direct electron transfer greatly improved the sensitivity for biosensing of hydrogen peroxide without need of mediator. The myoglobin/HMS based biosensor could detect hydrogen peroxide down to 62 nM [30]. The gold nanoparticle modified electrode could provide biocompatible surface for immobilization of cancer cells, which led to amplified electrochemical signal from the oxidation of cytoplasm guanine for electrochemical study of exogenous effect [36]. By immobilizing cancer cells on carbon nanotube-modified electrode to accelerate guanine oxidation, a method for electrochemical antitumor drug sensitivity test was also developed [37].

Carbon nanomaterials such as carbon nanotubes (CNTs) show the electrocatalytic behavior to low the activation energy of oxidation of biomolecules [38]. Thus, an amperometric biosensor for 3,4-dihydroxyphenylacetic acid was developed [39]. By accelerating the electron transfer between guanine or adenine residues of signal strand DNA (ssDNA) as well as adenine residues of RNA and CNTs modified electrode, a method for rapid sensitive detection of DNA or RNA was designed using a composite screen-printed carbon electrode [40]. The advantages of convenient fabrication, low-cost detection, short analysis time and combination with nanotechnology for increasing the sensitivity made the subject worthy of special emphasis. Soluble carbon nanofiber (CNF) possesses excellent electrical conductivity, unique structural and catalytic properties, high loading of biocatalysts, and good stability. A series of amplified biosensing strategies for highly sensitive detection of important biological compounds such as NADH as well as dehydrogenase substrates [41], glucose [42], ethanol [43], acetylthiocholine [44], phenol [45] and hydrogen peroxide [46]. Compared to single-walled CNTs, CNF has a much larger functionalized surface area to immobilize the enzyme, thus showed better performance for biosensing [47]. In 2009, two kinds of three-dimensional ordered graphitized mesoporous carbon GMC-6 (pore diameter 6 nm) and GMC-13 (pore diameter 13 nm) were also prepared for the immobilization of hemoglobin and the construction of electrochemical biosensors [48]. GMC-6 offered significant advantages over GMC-13 and CNTs in facilitating the electron transfer of entrapped protein and improving the performance of the fabricated biosensors [49].

2.2. Electrochemical immunoassay based on accelerated electron transfer

Immunoassay is usually performed by competitive or sandwich formats. Both of them utilize a label or tag as the signal probe for quantifying the antigen–antibody reaction. By accelerating the electron transfer between HRP as the label and electrode surface, a concept called as reagentless immunosensing was proposed for the determination of protein biomarkers [50]. In the presence of titania gel nanoparticles for immobilization of CA 125 antigen, the direct electrochemistry of HRP labeled to antibody was achieved. The HRP-labeled antibody could be bound to electrode surface upon a competitive immunoreaction of between the limited amount of HRP-labeled CA 125 antibody with CA 125 in sample solution and the immobilized CA 125, which led to the current decrease for immunoassay of CA 125. A similar reagentless amperometric immunosensor was also constructed for determination of human serum chorionic gonadotrophin (HCG) by immobilization of HCG with titania sol–gel on an electrode and the direct electrochemistry of HRP labeled to HCG antibody (HRP-anti-HCG) [51]. After gold nanoparticles were doped in the titania gel membrane via a vapor deposition method, a porous and homogeneous composite architecture without the aggregation of the immobilized protein molecules was obtained, and the direct electrochemistry of encapsulated HRP-labeled hCG antibody showed better performance in reagentless electrochemical immunoassay [52]. The formation of immunoconjugate by a simple one-step immunoreaction between hCG in sample solution and the immobilized HRP-anti-hCG introduced a barrier of direct electrical communication between the immobilized HRP and the electrode surface, which led to the signal decrease for hCG analysis.

The accelerated electron transfer between HRP and electrode can also be achieved in a designer organically modified silicate (ormosil) sol–gel [53]. The synthesized ormosil architecture provided a three-dimensional ordered nanoporous structure with high electrical conductivity, excellent mechanical stability and good hydrophobicity for retaining the activity of immobilized enzyme labeled immunocomponent [54]. By immobilizing HRP-labeled carcinoembryonic antigen (CEA) antibody (HRP-anti-CEA) or HRP-anti-hCG in the architecture, the proposed immunosensors showed a surface-controlled electrode process attributed to the oxidation of HRP with a rate constant of $5.94 \pm 0.40\,s^{-1}$ and $15.8 \pm 3.8\,s^{-1}$, respectively, which decreased upon the formation of immunocomplex in sample solution with a simple one-step immunoreaction.

To achieve the fast electrochemical immunoassay, a strategy of electric field-driven incubation was developed with a reagentless immunosensor prepared by immobilizing HRP-labeled antibody in a designed gel matrix [55]. The incubation for immunorecognition could be completed within 2 min. The immobilized HRP showed excellent direct electrochemistry, and the detection procedure was greatly simplified by directly monitoring the sensitive electrochemical signal of HRP upon the immunoreaction. Using α-fetoprotein as a model the linear detection range was from 0.02 to 2.0 ng mL^{-1}. The electric field-driven incubation could be for multianalyte electrochemical immunoassay by immobilizing respectively HRP-labeled antibodies modified gold nanoparticles in biopolymer/sol–gel modified electrodes to obtain direct electrochemical responses of HRP [56,57]. As shown in Fig. 1, the responses decreased due to increasing spatial blocking and impedance upon the formation of immunocomplexes. At a driving potential of 0.5 V, the incubation process could be accomplished within 2 min. This method could simultaneously detect CA-153, CA-125, CA-199 and CEA ranging from 0.084 to 16, 0.11 to 13, 0.16 to 15 U mL^{-1} and 0.16 to 9.2 ng mL^{-1} with a detection time of less than 5 min, respectively [56], which showed higher sensitivity and shorter analytical time than those of 0.4 to 140, 0.5 to 330, 0.8 to 190 U mL^{-1} and 0.1 to 44 ng mL^{-1} in a detection time of 40 min without electric field-driven incubation [57].

The electron transfer between HRP and electrode can be accelerated with single-walled carbon nanohorns (SWNHs). With a competitive immunoassay format, the analyte molecules could be easily covalently bound to SWNHs to functionalize the carbon nanoparticles for preparation of the immunosensor [58]. The abundant carboxylic groups on the cone-shaped tips of SWNHs enhanced the immobilization capability of analyte and provided a three dimensional recognition of HRP-antibody to the binding sites of analyte antigen. Based on the direct electrochemistry of HRP, a sensitive electrochemical immunoassay method was proposed for MC-LR detection in environmental samples, which showed good performance with high sensitivity, a wide linear range, acceptable precision and fabrication reproducibility, and excellent stability.

2.3. Stripping analysis for electrochemical biosensing

Electrochemical stripping analysis of nanoprobes containing metal element can greatly enhance the detection sensitivity of specific biosensing by bringing massive signal species to single recognition event. The nanoprobes can be prepared by

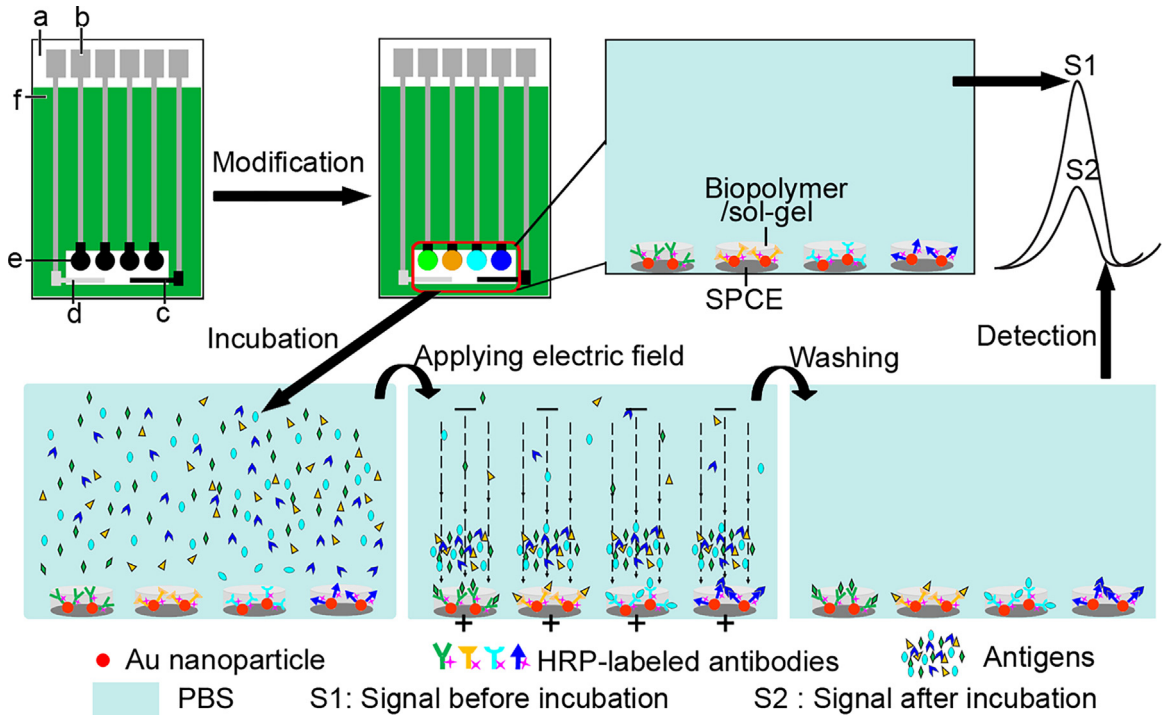

Fig. 1. Schematic representation of ECIA and the electrochemical multiplexed immunoassay with an electric field-driven incubation process. (a) Nylon sheet, (b) silver ink, (c) graphite auxiliary electrode, (d) Ag/AgCl reference electrode, (e) graphite working electrode, (f) insulating dielectric.
Adapted with permission from Ref. [56]. Copyright 2008 American Chemical Society.

functionalizing metal nanoparticles such as Au nanoparticles [59–62], Ag nanoparticles [63–65] and metal sulfides or tellurides [66–68] with recognition molecules such as oligonucleotides, antigen/antibody, aptamer, avidin/biotin, etc. Such protocols rely on capturing the metal element-contained nanoprobes to the target immobilized on electrode or support surface via single recognition couple and then use anodic-stripping voltammetry for measuring the metal tracer electrochemically. The target immobilization can be directly accomplished via the specific recognition to the immobilized capture molecules. The stripping analysis can detect picomolar and sub-nanomolar levels of DNA or protein target. For example, an electrochemical method was employed for the Au-nanoparticle-based quantitative detection of the 406-base human cytomegalovirus DNA sequence (HCMV DNA) [60]. The HCMV DNA was immobilized on a microwell surface and hybridized with the complementary oligonucleotide-modified Au-nanoparticle. The resulting surface immobilized Au nanoparticle double-stranded assembly was treated with HBr/Br_2 for oxidative dissolution of the gold particles, and the solubilized Au^{3+} ions were then electrochemically reduced to accumulate on the electrode and subsequently determined by anodic stripping voltammetry. As non-linear mass transport of the ions, and the release of a large number of Au^{3+} ions upon the dissolution of the particle associates with a single recognition event, this method enabled the detection of the HCMV DNA as low as 5×10^{-12} M. Further sensitivity enhancement can be obtained by catalytic enlargement of the gold tracer in connection to nanoparticle-promoted precipitation of gold [59] or silver [64]. For example, an ultra-sensitive technique for the electrochemical detection of the mutated BRAF gene associated with papillary thyroid carcinomas was developed by immobilizing a biotinylated 30-nucleotides probe DNA in a streptavidin-modified 96-well microtiter plate and hybridizing the biotinylated target DNA with the immobilized probe DNA [69]. After streptavidin-labeled gold nanoparticles were added, a nanoparticle enlargement process was performed using gold ion solution and formaldehyde reductant, and the gold nanoparticles were then dissolved in bromide to performed square wave stripping voltammetric measurement. This method could detect the target DNA in the concentration of 0.52–1300 aM. The limit of detection was approximately three orders of magnitude lower than that obtained using the similar method without gold amplification process.

The electrochemical stripping analysis of metal nanoparticles can be directly performed in KCl/HCl solution without need of dissolving them in bromide. For example, using gold nanoparticle to label antibodies, a simple, sensitive and low-cost multiplexed immunoassay was proposed by combining a disposable immunosensors array prepared with capture antibodies. Upon a sandwich immunoreaction, the analytes were bound to the corresponding capture antibodies for further capture of the gold nanoparticle labeled antibodies, and gold nanoparticles could be electrooxidized in 0.1 M HCl to produce $AuCl_4$ for voltammetric detection [70]. The immobilized gold nanoparticles can be further amplified via simple and convenient host-guest reaction between thio-β-cyclodextrin (SH-β-CD) functionalized gold nanoparticles labeled to signal antibody and SH-β-CD end-functionalized gold nanorods (AuNRs) [71]. The built end-to-end AuNRs superstructure showed excellent performance for the signal amplification in connection with the electrochemical biosensing by preoxidation at +1.60 V for 60 s and then voltammetric analysis of gold element from +0.68 to +0.28 V in 0.1 M HCl solution.

The sensitivity enhancement via silver deposition and then direct stripping can be achieved with both gold [72] and silver [73] nanoparticles labeled antibodies to catalyze the deposition reaction. For example, a streptavidin functionalized silver nanoparticles enriched carbon nanotube (CNT/Ag NPs) was designed as trace tag through one-pot in situ deposition of Ag NPs on carboxylated CNT and then functionalization with streptavidin to link biotinylated signal antibodies [73]. After sandwich-type immunoreaction on a disposable immunosensor array, numerous Ag NPs were captured

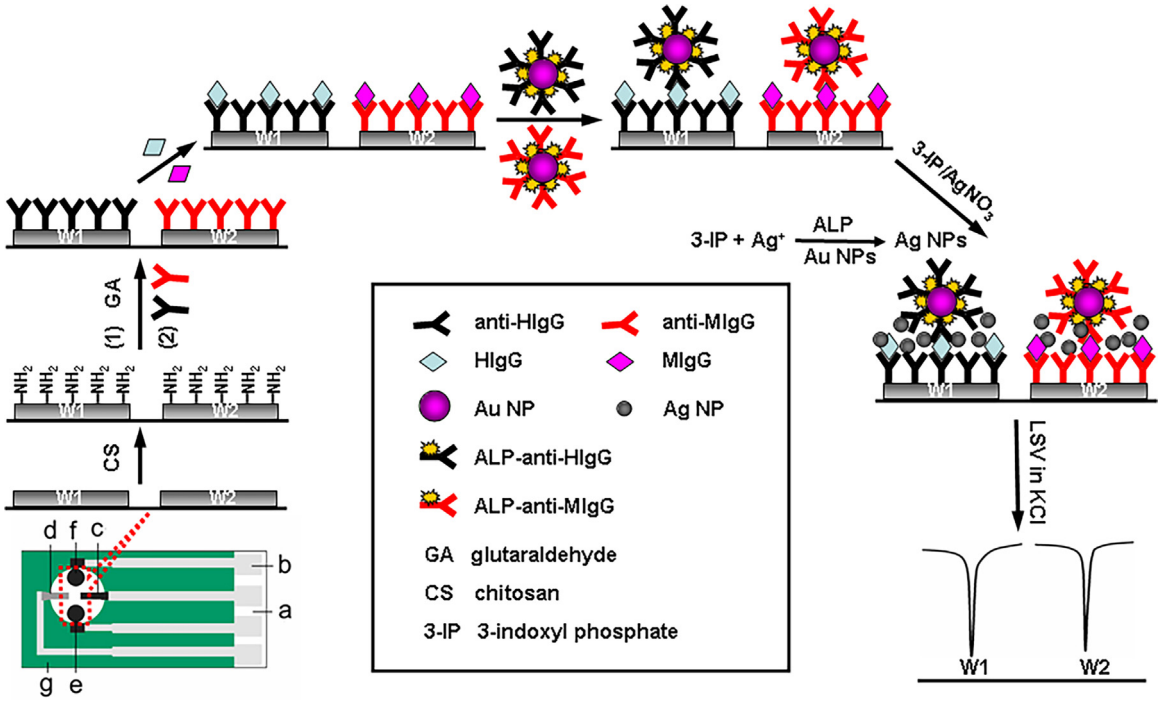

Fig. 2. Schematic representation of preparation of immunosensor array and detection strategy by sandwich-type immunoassay and linear sweep voltammetric stripping analysis of enzymatically deposited Ag NPs. (a) Nylon sheet, (b) silver ink, (c) graphite auxiliary electrode, (d) Ag/AgCl reference electrode, (e) W1, (f) W2, and (g) insulating dielectric.

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to every single immunocomplex, which were further amplified by a subsequent Ag NPs-promoted deposition of silver from the silver enhancer solution to obtain the sensitive electrochemically stripping signal of Ag NPs with linear sweep voltammetry from −0.15 to 0.25 V in 1.0 M KCl solution. Using carcinoembryonic antigen and α-fetoprotein as model analytes, this multiplexed immunoassay method showed wide linear ranges over four orders of magnitude with the detection limits down to 0.093 and 0.061 pg mL^{-1}, respectively. The silver deposition process could be catalyzed by both alkaline phosphatase (ALP) and Au nanoparticles to amplify the detection signal with ALP-labeled antibody functionalized gold nanoparticles (ALP-Ab/Au NPs) [74]. As shown in Fig. 2, the enzyme-Au NP catalyzed deposition of silver nanoparticles at disposable immunosensor array contained the hydrolysis of 3-indoxyl phosphate to produce an indoxyl intermediate for Au NP catalyzed reduction of Ag^+, and the deposited silver was then measured by anodic stripping analysis in KCl solution.

Wang's group used encoding nanoparticles (cadmium sulfide, zinc sulfide, copper sulfide, and lead sulfide) to demonstrate that inorganic nanocrystals or quantum dots (QDs) offer an electrodiverse population of electrical tags for multiplexed bioanalysis of DNA targets [66], SNPs [67], and antigens [68]. The multi-target electrical detection capability was coupled to the amplification feature of electrochemical stripping transduction to yield fmol detection limits. They also designed a QD/aptamer-based ultrasensitive electrochemical biosensor for a simple single-step displacement assay involving the coimmobilization of several thiolated aptamers to detect multiple protein targets [75]. Such electronic transduction of aptamer–protein interactions was extremely attractive for meeting the low power, size, and cost requirements of decentralized diagnostic systems. By the assembly of QDs on polystyrene-co-acrylic acid microbeads to prepare the DNA probes and the streptavidin-biotin binding, a detection method of DNA hybridization process was achieved by square wave voltammetry of Cd^{2+} after the dissolution of CdTe tags with HNO_3, which gave rise to a detection limit of 0.52 fmol L^{-1} and a wide dynamic range spanning 5 orders of magnitude [76].

The stripping analysis can be combined with DNA recognition or molecular biological protocols to further improve the sensitivity of DNA or protein detection. For example, with the use of RCA technique, a cascade signal amplification strategy was proposed for detection of protein target at ultralow concentration [77]. In this assay, the ultrasensitive detection was achieved by combining the RCA technique with oligonucleotide functionalized QDs, multiplex binding of the biotin-strepavidin system and anodic stripping voltammetric measurement. The RCA product containing tandem-repeat sequences could serve as excellent template for periodic assembly of QDs, which presented per protein recognition event to numerous quantum dot tags for electrochemical readout. Both the RCA and the multiplex binding system showed remarkable amplification efficiency, while very little nonspecific adsorption and low background signal. Using human vascular endothelial growth factor (VEGF) as a model protein, the designed strategy could quantitatively detect protein down to 16 molecules in a 100 μL sample with a linear calibration range from 1 aM to 1 pM and was amenable to quantification of protein target in complex biological matrixes. After this strategy was coupled with nicking endonuclease and assistant DNA helped target recycling on a molecular beacon (MB) modified electrode, the electrochemical readout of attached QDs as signal tags could detect DNA down to attomolar level (11 aM) with a linear range of 6 orders of magnitude (from 1×10^{-17} to 1×10^{-11} M) [14]. The cascade signal amplification strategy achieved high sensitivity and specificity, thus it seems to be a powerful tool for proteomics research and clinical diagnostics.

Recently, proximity hybridization regulated immunoassay that relies on simultaneous recognition of target protein by a pair of affinity probes and subsequently induces DNA hybridization to convert protein-recognition event into DNA detection has attracted considerable interest due to the introduction of a variety of nucleic acid amplification techniques such as PCR and RCA to

enhance the sensitivity [78,79]. Several amplified electrochemical immunosensing strategies based on proximity hybridization have been developed in Ju's group [80–83]. By combining the target-induced proximity hybridization on single-stranded DNA-gold nanoparticels@graphene oxide modified carbon electrode and electrochemical stripping analysis of silver nanoparticles, a simple and disposable electrochemical immunosensor was developed for sensitive and selective detection of protein biomarker [84]. In the presence of target protein and two DNA-labeled antibodies, the proximate complex formed in homogeneous solution could hybridize with the assembled DNA to take away AuNPs, which decreased AuNPs-catalyzed deposition of AgNPs on immunosensor surface, and thus the anodic stripping signal. The proposed method avoided the interference of dissolved oxygen and could CEA down to 3.9 pg mL^{-1}.

3. Functional nanoprobes for amplified biosensing

3.1. Nanoprobes as signal emitters

Nanotechnology offers unique opportunities for creating highly sensitive innovative biosensing devices and ultrasensitive bioassays. The unique optical, photophysical, electronic and catalytic properties of nanoparticles turn them into ideal labels for biorecognition and biosensing processes. For example, the unique plasmon absorbance features of metal nanoparticles and the tunable fluorescence properties of semiconductor nanoparticles have been widely used for DNA and antibody–antigen analyses. Besides the electrochemical stripping analysis of nanoprobes, they can act as different signal emitters for amplified biosensing.

Key challenges in current fluorescent sensing and imaging are to improve the sensitivity (brightness), selectivity and long-term stability of the sensing and imaging systems, particularly in complex and hostile environments, as well as applications for disease management, and pathogen identification [85]. The high and stable fluorescence intensity of QDs as well as carbon dots has resulted in improved sensitivity of fluorescent detection [86]. Moreover, as electrochemical stripping analysis, the improved sensitivity can be attributed to numerous signal molecules contained in a single nano-size tag. Thus, QDs have been used as excellent fluorescent labels for biological imaging, sensing, and diagnostics [87,88]. The broad absorption and narrow emission spectra of the QDs have also made them excellent donors of fluorescence resonance energy transfer (FRET) for QDs-based biosensing [89]. The FRET between QDs and graphene-like 2D nanomaterials leads to the fluorescence quenching. By using of the different interaction of these nanomaterials with ssDNA and dsDNA, the FRET has been extensively used for amplified sensing of biomolecules. For example, Dong et al. [90] designed an effective sensing strategy for detection of DNA by FRET from QDs to graphene oxide (GO) using molecular beacon modified QDs to recognize the target analyte. The strong interaction between MB and GO led to the fluorescent quenching of QDs. Upon the recognition to target, the distance between the QDs and GO increased, and the interaction between target-bound MB and GO became weaker, which significantly hindered the FRET and thus increased the fluorescence of QDs. By substituting the MB with aptamer, this strategy could be conveniently extended for detection of other biomolecules, thus opened new opportunities for sensitive detection of biorecognition events. Another fluorescent method can be developed by releasing the metal ions from QDs to sensitize the fluorescent emission of Rhod-5N. Using CdTe QDs functionalized silica nanosphere as label, this strategy was coupled with isothermal exponential amplification (IEA) to design an ultrahighly sensitive fluorescent method for detection of target DNA [91]. Molecular beacon as recognition probe was immobilized on a well plate to recognize target DNA, and the stem part hybridized with a primer to initiate the polymerization of DNA strand, which led to the release of target to open another MB molecule and start next cycle of strand-replacement polymerization. Meantime, the formed double-strand DNA was recognized by nicking endonuclease, leading to an endonuclease-based strand-replacement polymerization, which produced DNA trigger to open more MB. Upon a dissolving process, the Cd^{2+} sensitized fluorescence of Rhod-5N could detect target DNA ranging from 10^{-17} to 10^{-11} mol L^{-1} with a detection limit down to ~50 copies.

The plasmon absorbance feature of Au nanoparticles can be used for signal amplification [92]. For example, the interaction of cationic Au nanoparticles with 16s rRNA hybridized on the peptide nucleic acid probe-immobilized SPR sensor chip showed a detection limit of 58.2 ± 1.37 pg mL^{-1} for *Escherichia coli* rRNA without PCR amplification [93]. A oligonucleotide-capped Au nanoparticle designed for sandwich assay of oligonucleotides or polynucleotides with a flow injection SPR showed a detection limit of 2.1×10^{-20} mol for 39-mer target DNA [94]. Using longitudinal plasmonic resonance of gold nanorods for ultra-sensitive SPR biosensing with functionalized gold nanorods as amplification labels, the sensitivity enhancement could be maximized due to the electromagnetic interaction between the nanotag and the sensing film [95]. A fiber-optic biosensor based on a localized surface plasmon coupled fluorescence (LSPCF) system consisting of a biomolecular complex in a sandwich format of antibody/antigen/Cy5-antibody-Au nanoparticle could detect mouse immunoglobulin G (IgG) as low as 1 pg mL^{-1} (7 fM) during the biomolecular interaction of the IgG with anti-mouse IgG [96]. Based on Au nanoprobes, a one-step, washing-free and amplification-free assay for protein analysis by dynamic light scattering (DLS) technique was developed to determine the concentration of target protein by analyzing the level of Au aggregation caused by antibody–antigen interactions using DLS [97]. Recently, the fluorescence emission of gold nanoclusters has also attracted intention. For example, an OVA-stabilized fluorescent gold nanocluster nanoprobes has been constructed for sensing glucose [98].

The electrochemiluminescence (ECL) is the luminescence generated by relaxation of exited state molecules that are produced during an electrochemically initiated reaction. Since the ECL study of nanoparticles was first reported in 2002 for Si NPs [99], the ECL of nanoparticles has been studied [100] and applied in amplified biosensing [101–103]. Ju's group mixed CdSe hollow spherical assemblies in carbon paste to present a method for studying the ECL behaviors of nanoparticles in aqueous system [104], and immobilize CdSe QDs on paraffin-impregnated graphite electrode to develop the first QDs-based chemical sensor for detection of H_2O_2 ranging from 2.5×10^{-7} to 6×10^{-5} M [105]. They used this electrode to couple QDs with an enzymatic reaction to report a simple strategy for the first application of QDs in ECL biosensing, which led to the first QDs-based ECL biosensor for detection of oxidase substrates [106]. The biocompatible shell structure of QDs improved greatly the stability of the coimmobilized biomolecules such as glucose oxidase (GOD) for preparation of biofunctionalized QDs film, thus produced a sensitive and stable ECL signal for detection of glucose at physiological pH. After elucidating the detailed ECL process of thioglycolic acid-capped CdSe QDs film/H_2O_2 aqueous system, they thought the intermediate $OH^{\bullet}$ radical was a key species for producing holes injected QDs for $1S_e$-$1S_h$ ECL emission, thus proposed a strategy for ECL sensing of the scavengers of hydroxyl radical [107].

The ECL emission generally involves two mechanisms: annihilation and coreactant mechanisms. The overwhelming majority of QDs-based ECL applications concern coreactant ECL, including oxalate system, tri-n-propylamine system, and H_2O_2 or $S_2O_8{}^{2-}$ system. Ju's group demonstrated a conreaction, $SO_3{}^{2-}$, for anodic ECL

of CdTe QDs, and thus proposed an ECL energy transfer mechanism for detection of catechol derivatives [108] and an enzymatic cycle-amplified biosensing strategy for highly sensitive bioanalysis [109]. They then made use of self-produced coreactant from oxygen reduction to propose an immunoassay method [110]. By the enzymatic reduction or electrocatalytic reduction to consume dissolved oxygen or coreactant, which lowed the ECL emission, several ECL immunosensors for protein detection [110–114] and DNA biosensors [115] were thus designed. The ECL quenching could also be achieved by chemical reaction [116], electrochemical reaction [117] and electron-energy transfer [118], which led to different biosensing strategies.

The low-potential ECL emission is an efficient way to avoid the interference of electroactive species coexisted in samples. Liu et al. [119] developed a method for the formation of surface traps on QDs by bidentate chelation, with which they proposed an analyte competition strategy for cation detection [120] and an ECL biosensor for glucose [121]. Through the surface passivation, another QDs, phenol formaldehyde resin@CdS quantum dots, was synthesized for low-potential ECL biosensing [122]. High electron transfer efficiency of some nanomaterials such as titania dioxide nanotube is also an efficient method to achieve the low-potential ECL emission [123].

The amplified ECL emission can be achieved by using nanomaterials such as carbon nanotubes (CNTs) [124], nitrogen-doped carbon nanotubes (NCNTs) [125], carbon nanospheres [126], reduced graphene oxide (ERGO) [127] to accelerate the electron transfer and reduce the injection barrier of electrons to the QDs. The composition of CdSe QDs with NCNTs greatly enhanced the cathodic ECL emission and shifted positively the starting potential to produce the ECL response. The ECL intensity was 3-fold that from multi-walled CNTs composited CdSe QDs, and the starting potential was also 0.22 V more positive than that of MWCNTs composited CdSe QDs [125]. After the electrochemical reduction of GO by scanning the potential from 0 V to −1.2 V in 0.1 M pH 7.0 phosphate buffer solution, the restoration of structural conjugation led to 4.2-times and 178.9-times increased ECL intensity of the QDs/ERGO modified electrode as compared with intrinsic QDs and QDs/GO modified electrode due to the adsorption of dissolved O_2 on ERGO and the facilitated electron transfer, respectively [127]. By synthesizing QDs in polymers to produce nanocomposite signal probe, the ECL intensity can be further enhanced. For example, the nanocomposite of poly(amidoamin) (PAMAM) dendrimer and CdS QDs prepared on electrode surface showed 55-fold enhanced ECL compared with that of QDs film without dendrimer [128]. By covalently coupling the nanocomposite of CdS QDs-PAMAM dendrimer to amino group of DNA probe for signal amplification of ECL measurements, an ultrahighly sensitive protocol for detection of near single DNA molecule with a linear range of 7 orders of magnitude was developed [129].

Recently, new ECL emitters with low toxicity, high efficiency, good stability and acceptable repeatability for ECL detection of heavy metal ions, small biomolecules, proteins and nucleic acids have attracted extensive attention. The ECL activity of metal nanoclusters and carbon nanocrystals suggests their promising applications in the development of new types of biosensors [130,131]. Graphite-like carbon nitride (g-C_3N_4) has been proved to be an effective ECL emitter [132] and exhibits greatly improved optical and electrical behaviors in cathodic ECL emission after it is exfoliated into carbon nitride nanosheets (CNNS) [133]. In the presence of triethylamine (Et_3N) as a coreactant, the electro-oxidation of g-C_3N_4 produces the excited state and anodic ECL emission [134]. Due to the annihilation of oxidation product $Et_3N^{\bullet}$ radical by the oxidation product of dopamine as analyte ($DA^{\bullet+}$), the ECL emission decreases, which leads to a facile "signal-off" strategy for the detection of dopamine. Through the adsorption of hemin-labeled ssDNA on CNNS modified electrode to electrocatalyze the reduction of dissolved oxygen for inhibiting the formation of coreactant H_2O_2, a "signal-on" strategy for specific detection of DNA can be developed [135]. The ECL emission of conjugated polymer nanoparticles or polymer dots (Pdots) was observed in 1994 [136]. The low solubility and rather positive oxidation potentials for producing the anodic ECL emission of conjugated polymer in aqueous media limit their application in biosensing, though the ECL behaviors of three basic types of conjugated polymers such as poly(phenylenevinylene) (PPV) [137], poly(3-hexylthiophene) [138], and poly(fluorene) [139] have been studied. Feng et al. used a functional monomer, silole, with low LUMO to design a donor–acceptor strategy for preparation of a conjugated polymer, silole-containing polymer (SCP) and further synthesize conjugated polymer Pdots for low-potential ECL emission and biosensing [140]. In order to circumvent low ECL efficiency, this group further integrated the luminescent feature and carrier function of Pdots to design a double-enhanced ECL mechanism by encapsulating a large amount of $Ru(bpy)_3^{2+}$ in Pdots [141]. The high loading of $Ru(bpy)_3^{2+}$ in Pdots led to multiplex ECL emission for single target molecule, and the resonance energy transfer (RET) from excited Pdots to $Ru(bpy)_3^{2+}$ further increased the emission intensity. Thus an ECL method for single-nucleotide polymorphism detection was proposed using DNA functionalized RuPdots as nanoprobe for signal acquisition and a ligase reaction to endue the method with specificity. The analytical performance of the proposed ECL method for detection of mutant *KRAS* gene as a model target demonstrated promising potential of the RuPdots in sensitive biodetection.

Photoelectrochemical (PEC) biosensing based on the interaction of the recognition event with PEC reaction of nanomaterials is another popular hotspot in this field [142]. It employs current as a detection signal with light as an excitation source and can be performed via reactant determinant-, electron transfer- and energy transfer-based sensing strategies [143]. Some PEC materials such as TiO_2, SnO_2, CdS and CdSe semiconductor nanoparticles have been used for biosensing purpose. Highly sensitive PEC sensor based on SnO_2 nanoparticle-modified indium tin oxide (ITO) electrode has been developed for the detection of adenosine triphosphate in the extracts of cancer cells [144]. The photoelectrochemistry of CdS nanoparticles has been used for detection of tyrosinase activity [145]. The integration of Au-doped TiO_2 nanotubes with acetylcholinesterase has led to a rapid and valid PEC approach for the determination of acetylcholinesterase inhibition with an illumination at 253.7 nm [146]. The photo-current conversion efficiency of TiO_2 nanoparticles in molecular electronics and PEC devices can be improved with porphyrins [147]. Tu et al. synthesized a functional TiO_2 nanoparticles using water-soluble [meso-tetrakis(4-sulfonatophenyl) porphyrin] iron(III) monochloride (FeTPPS) via the dentate binding of TiO_2 nanoparticles with sulfonic groups of FeTPPS [148]. The stable PEC response of the nanoparticles could be sensitized through an electron transfer process from biomolecule to porphyrin and then to the illuminated TiO_2, leading to an application of porphyrin functionalized TiO_2 nanoparticles as PEC biosensing platform [148,149]. Under irradiation, QDs can generate photoelectron from its precursor, the exciton [150], which is spatially restricted by the quantum confinement and thus leads to the phenomenon of quantum efficiency exceeding 100% [151]. The circumstantial alternation can sensitively change the quantum states of the QDs, which reflects in the exciton states, leading to the status change of the photoelectron. Thus Wang et al. proposed a concept of quantum photoelectric effect to describe the photocurrent response mechanism of QDs [152]. Based on the interaction of copper ion with QDs to produce the trapping sites of exciton, they developed a visual method for "signal off" detection of copper ion with a wide concentration range and a "signal on" immunoassay method for protein detection by labeling the

secondary antibody with QDs and simply immobilizing the capture antibody on a glass substrate. They also presented a concept of energy resonance absorption to quench the photocurrent of QDs, which led to new methodology for biosensing [153]. The coupling of QDs with different recognition events such as aptamers to their targets based on target-dependent aptamer conformational conversion [154], immunological reaction [155] and DNA hybridization [156] expands the target range of PEC biosensing. Furthermore, the recognition can introduce chemiluminescence system to act as an excitation source, which avoids the aid of physical light source [155,156]. The photoelectrochemical activity can be improved by functionalizing QDs with porous ZnO nanosheets [157] or carbon nitride nanosheets [158], which led to a methodology for design of hydrogen peroxide-related biosensors by the formation or consumption of hydrogen peroxide [157]. The surface plasmon resonance of gold nanoparticles can also enhance the PEC response for sensitive biosensing [159]. By combining with ratiometric assay [160], proximity assay [161] and catalytic hairpin assembly [162], several strategies have recently been developed for amplified biosensing.

3.2. Nanoprobes with catalytic activity

The catalytic functions of enzymes have been extensively applied in biosensing fields. However, their stability is limited due to easy denaturation and leakage of biomolecules during their storage and immobilization procedure. Therefore, the syntheses of artificial biomolecules or nanostructured materials with highly catalytic properties and a wide range of practical applications are becoming a significant field for different purposes. This section mainly summarizes the catalytic activity of nanomaterials or nanoprobes for amplifying the biosensing signals.

Fe_3O_4 nanoparticles possess intrinsic enzyme mimetic activity similar to that found in natural peroxidases [163]. Its ability to catalyze the oxidation of organic substrates can produce a color change, which is frequently used as a detection tool for different targets. A Fe_3O_4/chitosan modified glassy carbon electrode has been prepared for H_2O_2 and glucose detection [164]. This Fe_3O_4-based electrode shows the advantages of low applied potential, low background current and rapid response to H_2O_2. Furthermore, as an inorganic nanomaterial, it is expected to be more stable than the natural enzyme against solution pH and temperature. By functionalizing Fe_3O_4 nanoparticles with protein A, it can be used as a nanoprobe to label antibody for immunoassay [163].

FeS nanoparticles show similar enzyme mimetic activity as Fe_3O_4 nanoparticles [165]. It possesses a typical Michelis–Menten kinetics and good affinity to both H_2O_2 and 3,3′,5,5′-tetramethyl benzidine. The H_2O_2 sensor based on FeS shows better stability than that based on horseradish peroxidase when they are exposed to solutions with different pHs and temperatures. These excellent characters make the nanostructured FeS powerful for a wide range of potential applications as an “artificial peroxidase” in biosensors and biotechnology.

The electrocatalytic activity of $MoSe_2$ and black phosphorus nanoparticle as labels toward the hydrogen evolution reaction has also been used to produce sensitive electrochemical signal for magneto-immunoassays toward protein detection. The magneto-immunoassays display good selectivity and sensitivity [166,167].

The noncovalent combination of single- or multiwalled CNTs (SWNTs) with porphyrins can undergo fast electron transfer to show the electrocatalytic behavior toward reduction of oxygen [168]. A functional (SWNTs) assembled with water-soluble iron(III) meso-tetrakis(N-methylpyridinum-4-yl)porphyrin (FeTMPyP) via electrostatic interaction shows excellent electrolysis toward reduction of both oxygen and nitric oxide [169]. Similarly, the noncovalent functionalization of graphene with picket-fence porphyrin shows good dispersion and excellent electrocatalytic activity toward the reduction of chlorite, leading to highly sensitive amperometric biosensing with a detection limit of 2.4×10^{-8} mol L^{-1} at low applied potential [170]. In the presence of 1-butyl-3-methylimidazolium hexafluorophosphate ionic liquid, hydroxyferriprotoporphyrin functionalized single-walled CNTs can catalyze the electrochemical reduction of trichloroacetic acid (TCA), which leads to a highly sensitive and stable amperometric biosensor for TCA [171]. By combining the photocatalytic properties of porphyrin bound TiO_2 nanoparticles with single-walled carbon nanohorns (SWNHs), a sandwich nanohybrid of single-walled canbon nanohorn-TiO_2-porphyrin has been prepared for electrocatalytic reduction of chloramphenicol and highly sensitive and stable amperometric biosensing in neutral media [172]. At an applied potential of −0.56 V, the biosensor shows rapid response to chloramphenicol with a detection limit of 0.9 nM. Via the Fe-N axial coordination, water-insoluble picket-fence porphyrin can be assembled on nitrogen-doped multiwalled carbon nanotubes (CNx-MWNTs), which leads to the direct electrochemistry of porphyrin to form high-valent Fe(IV) porphyrin at low potential in neutral aqueous solution and produce excellent catalytic activity toward the oxidation of sulfite [173].

Recently, metal-organic frameworks (MOFs) with exceptional tenability have attracted considerable interest. The entrapping of functional molecules in MOF materials is a promising method for the design of functional MOFs and signal-transduction strategy. A series of porphyrin-encapsulated MOFs have been prepared using a template method and postsynthetic metal exchange, which remains the cavities for small molecules to reach the active sites of catalysis much like channels in heme proteins [174]. Through a one-pot incorporation of porphyrin FeTCPP into the cage of HKUST-1(Cu) MOF, a porphyrin-encapsulated MOF material has been synthesized to catalyze the oxidation of ophenylenediamine to 2,2′-diaminoazobenzene [175]. After functionalizing the FeTCPP@MOF with streptavidin, it can be used as a nanoprobes to conveniently label protein and DNA sequence via biotin-avidin recognition for design of different biosensing strategies. The similar strategy has been used to functionalize zirconium–porphyrin MOF with streptavidin for recognizing the product of target recycling on a triple-helix modified electrode in the presence of target DNA and Exo III, and obtaining the electrocatalytic oxidation signal for detection of DNA, which shows a detection limit of 0.29 fM [176]. The functionalized zirconium–porphyrin MOF can be combined with telomerase triggered extension, the assistant DNA 1–assistant DNA 2 duplex to switch into a hairpin structure immobilized on electrode surface for telomerase detection [177]. Besides the encapsulation of porphyrin in MOFs, noble-metal nanoparticles have been embedded in the MOFs. The high guest loading makes the nanoparticle/MOF composites possess high catalytic activity. For example, a platinum nanoparticles encapsulated MOF composite has been synthesized for the electrochemical detection of telomerase activity by the electrocatalysis of Pt NPs toward $NaBH_4$ oxidation and MOF functionalization with capture DNA [178].

In addition, the catalytic activity of noble-metal nanoparticles such as palladium nanocluster, AuPd bimetallic nanoprobes and platinum nanodendrite has been used for amplified immunosensing [179–181]. Through combining luminol/palladium nanocluster/graphene oxide labeled signal antibody with CdS QDs/Au nanoparticles and capture antibody modified electrode, a ratiometric ECL strategy regulated by the electrocatalysis of palladium nanocluster toward reduction of H_2O_2 and competition of luminol to the coreactant shows a detection limit of 0.62 pg mL^{-1} for immunosensing of CEA [179]. Using platinum nanodendrite functionalized graphene nanosheet as a non-enzymatic nanoprobe to label the signal antibody, the catalytic activity toward electroreduction of dissolved oxygen can be used to obtain amplified

immunosensing signal, which shows a good linearity in the concentration range of 1 pg mL^{-1} to 10 ng mL^{-1}, with a detection limit of 0.87 pg mL^{-1} for electrochemical detection of human immunoglobulin G (HIgG) [181].

3.3. Nanomaterials as carriers of signal molecules

In recent years, great efforts have been made to amplify the signal output by increasing the ratio of signal tag to specific recognition ligand. The simple and efficient method for this purpose is to use nanomaterials as carriers of signal molecule or enzyme to introduce more reporter molecules due to their high surface-to-volume ratio [182].

Enzymes are frequently used reporter molecules due to the highly efficient bioactivity of enzymatic reactions. The unique properties of the enzyme-functionalized nanoprobes enable the development of a large variety of signal transduction strategies for ultrasensitive biosensing. The early work covalently linked high ratio of HRP on carboxylated MWCNTs surface to prepare a nanoprobe for the sensitive immmunoassay of cancer biomarker [183]. Thus nanoprobes used about 90 catalytic labels for each binding event, thus greatly enhanced the sensitivity. Using Au nanoparticles functionalized CNT as carrier, Ju's group prepared a glucose oxidase (GOx) functionalized CNT/Au NPs nanoprobe for an ultrasensitive multiplexed electrochemical immunosensing [184]. As shown in Fig. 3, the immunosensor array was constructed through the stepwise assembly of colloidal Prussian blue (PB), Au NPs and captured antibodies on screen-printed carbon electrodes. The enzymatic reaction of the nanoprobe and electrocatalytic reduction of H_2O_2 by PB as a mediator provided sensitive electrochemical signal tracing of the immunosensors. Using carcinoembryonic antigen and α-fetoprotein as model analytes, the simultaneous multiplexed immunoassay method showed linear ranges of three orders of magnitude with the detection limits down to 1.4 and 2.2 pg mL^{-1}, respectively. A HRP functionalized graphene oxide nanoprobe has also been prepared to catalyze the oxidation of 4-chloro-1-naphthol substrate for impedimetric immunosensing [185]. The amplified inhibition of the enzymatic oxidation product to electron transfer of ferrocene indicator could be used for ultrasensitive amperometric immunoassay [186].

Au nanoparticles are widely used carrier for loading of reporter molecules due to the good biocompatibility [187]. A biotin and luminol functionalized gold nanoprobe has been designed and modified with streptavidin to label secondary antibody [188]. The chemiluminescence signal is produced by the oxidation of luminol molecules in the presence of $HAuCl_4$ as catalyst and H_2O_2 as oxidant, which shows about 40 times lower detection limit toward HBsAg relative to previous work [189]. In such case, the nanoparticles act as both a solid carrier to load a large number of reporters/markers and labels to amplify the chemiluminescence signal. In order to greatly enhance the chemiluminescence signal for imaging analysis, Ju's group prepared four tags by binding high loading ratio of HRP to detection antibodies to AuNPs [190]. By combining the AuNPs based tags with a chemiluminescence sensor array and a sensitive cooled low-light CCD, a highly sensitive chemiluminescence imaging immunoassay method was proposed for simultaneous detection of biomarkers. This group then designed a multilayer G-quadruplex/hemin DNAzyme wrapped gold nanoparticle (M-DNAzyme/AuNP) tag for ultrasensitive chemiluminescence imaging analysis of proteins [191]. The M-DNAzyme/AuNP tag was prepared by assembling high ratio of alkylthiol-capped signal DNA containing multiple G-quadruplex sequences to biotinylated DNA on AuNPs and then reacting with hemin to form multilayer hemin/G-quadruplex DNAzyme units. It was bound to the biotinylated secondary antibody of sandwich immunocomplex by biotin–streptavidin conjugation to catalyze a chemiluminescence reaction on a protein array, which produced strong chemiluminescence emission. By combining with a disposable protein array, an ultrasensitive and high-throughput multiplex CL immunoassay method was proposed for simultaneous detection of four cancer biomarkers, including fetoprotein, human chorionic gonadotrophin-β, carcinoma antigen 125, and CEA, with the limits of detection of 2.7×10^{-5} ng mL^{-1}, 1.1×10^{-5} IU mL^{-1}, 1.7×10^{-5} U mL^{-1}, 2.0×10^{-5} ng mL^{-1}, respectively. They also designed a DNA nano-polylinker with high loading of signal molecules as a three dimensional nanoprobe, which was prepared by rationally engineering dsDNA polymerization on

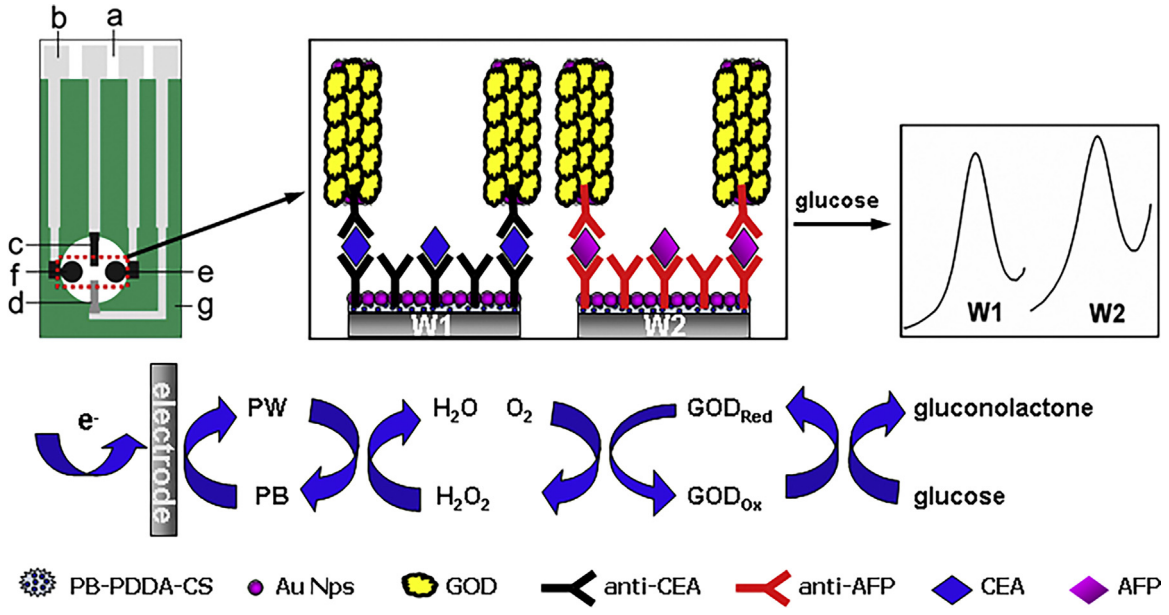

Fig. 3. Schematic representation of multiplexed electrochemical immunoassay with an immunosensor array and electrochemical response mechanism. (a) Nylon sheet, (b) silver ink, (c) graphite auxiliary electrode, (d) Ag/AgCl reference electrode, (e) W1, (f) W2, and (g) insulating dielectric.
Adapted with permission from Ref. [184]. Copyright 2009 American Chemical Society.

initiator DNA modified AuNP via a HCR with two kinds of FITC-labeled DNA hairpins [192]. The biotinylated core–shell nanoprobe was immobilized on the immunosensor surface, and the FITC molecules then bound enzyme labeled anti-FITC antibody to catalyze a silver deposition process via a classic sandwich type and a biotin-streptavidin affinity system. This method showed satisfactory results for linear detection range over 5 orders of magnitude for CEA with a detection limit of 1.2 fg mL^{-1}. Using Au NP to load many CdS NPs labeled linker DNA, a significant amplification for the detection of thrombin ranging from 1.0×10^{-15} to 1.0×10^{-11} M has been proposed [193]. Aptamers are immobilized on the Au NPs modified electrode for constructing the sandwich type detection strategy. The concentration of thrombin is monitored based on the concentration of dissolved Cd^{2+} formed in the dissolution of CdS by acid treatment and quantified by differential pulse voltammetry.

Mesoporous SiO_2 nanoparticles (MSN) and mesoporous carbon nanosphere are also suitable carriers for preparation of nanoprobes. Yang et al. [194] designed a MSN-based label by loading MSN with mediator thionine, HRP and secondary anti-human IgG antibody for sandwich-type immunoassay of human IgG in a range of 0.01–10 ng mL^{-1}. The covalent incorporation of $Ru(bpy)_3{}^{2+}$ in silica nanoparticles showed more than 1000-fold increase of the ECL signal, which suggested that this nanostructure as luminescent labels represents a very promising system for ultrasensitive bioanalysis [195]. By use of the flexible binding properties of DNA to seal electroactive molecules in the mesopores, a nanoprobe as regulated DNA bio-gate has been prepared to recognize the target-induced proximity hybridization product for release of the sealed electroactive molecules and electrochemical immunoassay [82]. By loading signal antibodies and high-content glucose oxidase on amino-functionalized silica nanosphere, an ultrasensitive multiplexed electrochemical immunoassay method was developed for the detection of tumor markers by combining a newly designed trace tag and a disposable immunosensor array, which could detect CEA and α-fetoprotein down to 3.2 and 4.0 pg mL^{-1} [196]. Besides these silica nanomaterials, carbon nanomaterials can be used as the carriers for the preparation of nanoprobes. A N-(4-aminobutyl)-N-ethyl isoluminol-functionalized graphene composite has been reported for utrasensitive label-free electrochemiluminescence immunosensor [197]. As an enzyme mimic, Prussian blue has been in situ deposited on mesoporous carbon nanosphere to load signal antibody and high-content glucose oxidase (GOD) for sensitive electrochemical immunoassay of IgG down to 7.8 pg mL^{-1} [198].

4. Functional nanoprobes for cytosensing

Cells are the basic units of life. Developing sensing platforms for probing the chemistry and physics in/at a living cell is one of the basic goals in understanding the intricate processes that ultimately contribute to life and life processes. Moreover, the identification of cellular signatures for early diseased cell detection is very significant in disease therapy. Thus ultrasensitive detection and imaging methods for cytosensing of related cellular functional molecules have emerged as a cornerstone solution by the combination of nanotechnology with chemistry, biology, physics, engineering and medicine [199]. This section describes the use of nanoparticles in the detection of analytes at or within a cell, including cell surface carbohydrates and some important cellular functional molecules.

4.1. In situ detection of cell surface glycans

Carbohydrates are involved in many biological processes and metabolism. Glycosylation of proteins affects the biological activity, lifetime, cellular uptake, and specificity of these proteins. Dynamic changes in the glycosylation status on carcinoma cell surfaces have been observed to play important roles in oncogenic transformation, cell differentiation, and metastasis. Thus sensitive analysis of carbohydrates on living cells in various events is keenly desirable for basic science advancement and clinical diagnostics. However, unlike oligaonucleotides and proteins, glycan chains are rarely expressed in a linear, unbranched fashion, and even when they are, such chains are often subject to various modifications, moreover, the biological consequences of altering glycosylation in various systems seem to be highly variable and unpredictable. Therefore, the methodological study for in situ detection of cell surface glycans has become increasingly important and emerged as the "new frontier" for elucidating fundamental biochemical processes and for identifying new pharmaceutical substances. Two types of nanomaterial-based signal amplification platforms have been developed for cell surface carbohydrate sensing: nanoscaffolds for immobilization of cells and electrochemical detection [200–204], and nanoprobes for carbohydrate recognition, signal transduction and signal amplification [205–209].

The nanomaterials or nanoscaffolds can increase electrode surface and accelerate electron transfer, and thus provides enhanced sensitivity for in situ electrochemical detection of cell surface carbohydrates by specific recognition of enzyme-linked lectins. The first electrochemical cytosensing strategy designed for this purpose used arginine-glycine-aspartic acid-serine (RGDS)-functionalized SWNTs to capture target cells on the electrode surface and HRP labeled concanavalin A (Con A) to recognize mannose groups on cells [200]. After specific recognition, the dual signal amplification of SWNTs and enzymatic catalysis produced an amperometric response for evaluation of the mannosyl groups on the cell surface. This work could be further expanded on an electrochemical cytosensor array for dynamic analysis of multiplex carcinoma cell surface glycans [201]. Using intact human leukemic K562 cell surfaces as model target, this method reflected the expression extent of different glycans on cell surfaces, which were in good agreement with flow cytometric results, thus could be used for effective monitoring of the dynamic variation of glycans on cancer cell surfaces during both drug inducement and erythroid differentiation of K562 cells. The target cells could also been captured on Au nanoparticles modified electrode for detection of P-glycoprotein on cell surface via its recognition to corresponding antibody and then the secondary alkaline phosphatase (AP) conjugated antibody to introduce AP onto the electrode-immobilized cells, which led to an amperometric response of 1-naphthyl phosphate [210]. This strategy was later expanded to investigate the expression extent of P-gp on HeLa cells [211] and BGC 823 cells [212].

The capture of cells on nanoscaffolds modified electrode results in the increase of electron transfer impedance. Thus electrochemical impedance spectroscopy (EIS) and ECL method can be used to monitor the change by the specific recognition of immobilized lectins to cell surface glycans. Using lectins functionalized single-walled carbon nanohorns (SWNHs) [202] or MWNTs [203] to modify the electrode, a label-free strategy for EIS analysis of cell surface glycan expression has been developed. The ECL strategy can be performed by immobilizing lectins functionalized CdSe QDs on electrode [204]. Upon the recognition of immobilized lectins to cell surface glycans, the electron transfer impedance increases, thus ECL intensity decreases.

Different nanoprobes have been designed for carbohydrate sensing, especially in situ cell surface carbohydrate monitoring. An earlier work for fabrication of nanoprobe was performed by using α-N-acetylgalactosamine (α-GalNAc) residues to CNTs and then binding the residues to *Helix pomatia agglutinin* (HPA) for recognition of cell surface glycan [205]. This work offered the opportunities for probing biological processes. Ju's group prepared a lectin-functionalized QD probe and a mannan carbohydrate

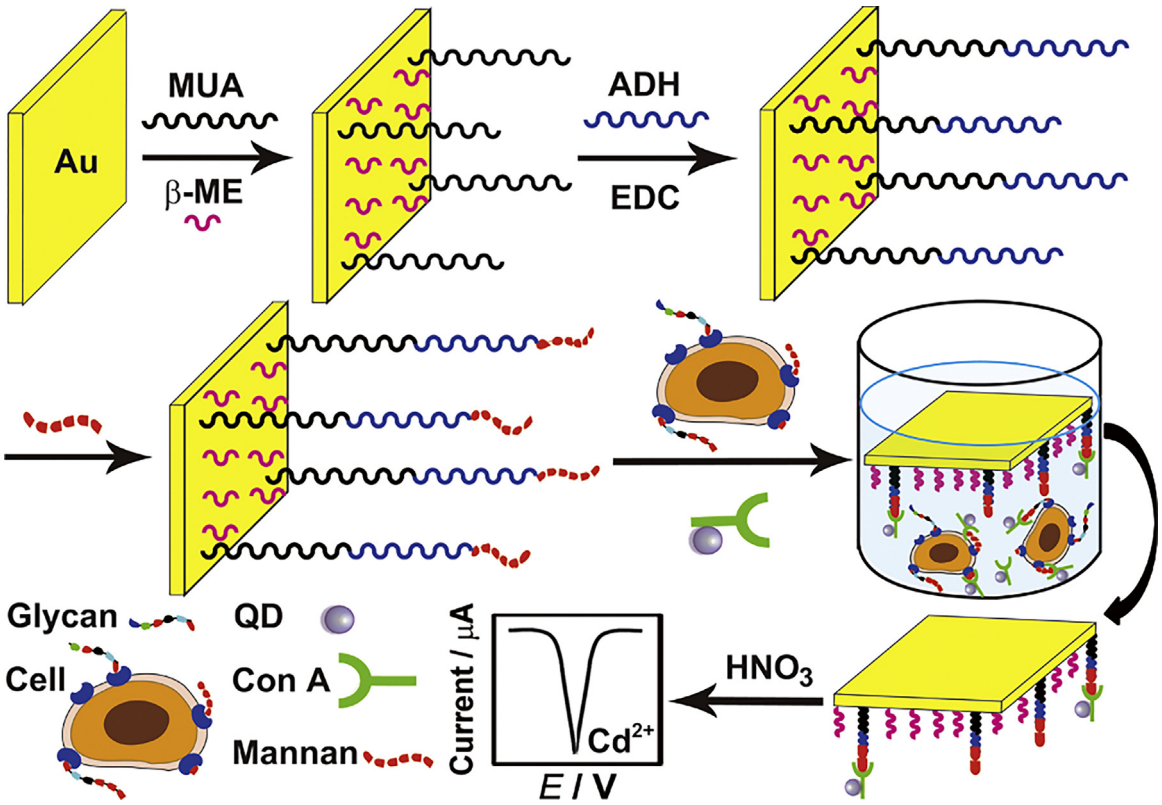

Fig. 4. Schematic representation of the monolayer fabrication and the competitive assay.
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monolayer for competitively recognizing the nanoprobe with cell surface carbohydrate [206]. This first-reported "one molecule-two surfaces competition format" allowed in situ analysis of cell surface mannose moieties. After the nanoprobe was captured by mannan monolayer, it was detected by stripping analysis technique to estimate the number of mannose moieties on cell surface (Fig. 4). By combining the signal amplification from stripping analysis with the competition format, this method could reach the detection limit of 10^2 cells/mL, and the average Con A binding capacity of single K562 cell could be estimated to correspond to 2.3×10^{10} mannose moieties. This group then prepared a dual-functionalized nanoprobe for highly sensitive and selective in situ evaluation of carbohydrates on living cells by integrating the specific carbohydrate recognition, enzymatic signal amplification of proteins on AuNPs and RGDS functionalized nanohorns to enhance the electrical connectivity, which shows a detection limit down to 15 cells [207]. An electrochemical lectin-probe, ferrocene-Con A was also prepared for in situ monitoring of cell surface glycan using the electroactive ferrocenyl group to reflect the probe binding extent by the cells [213]. The stripping analysis of nanoprobe can be combined with highly efficient chemoselective recognition for in situ electrochemical assay of cell surface sialic acids [214]. The C-7 position of sialic acids is firstly selectively oxidized by periodate to form aldehyde group for targeting biotin hydrazide through a highly efficient aniline-catalized bioorthogonal reaction. The avidin and 3-aminophenyl boronic acid (APBA) functionalized nanohorn probe is then bound to cell surface via biotin-avidin recognition to bind mannan-conjugated gold nanoparticles. The bound AuNPs can be released from the nanohorn probe by a competition reaction of fructose with APBA to perform the cathodic differential pulse voltammetric (DPV) scan after pre-electrooxidization of AuNPs at a disposable screen printed carbon electrodes.

Scanometric technique combining the advantage of colorimetric detection [215,216] with the array format has become hot detection method. Using glycan functionalized Au nanoparticles and cell surface glycan to competitively bind lectin in solution, a label-free scanometric approach has been designed to conveniently read out the number of cell surface carbohydrate groups by integrating the bioconjugation and aggregation of glyconanoparticles, silver signal amplification, and spot test [208]. This strategy can be used to develop another method for probing the surface composition by the competition between the two surfaces for specifically binding the receptor species to selectively transfer the biological information from a primary cell-adhered solid surface to a carbohydrate assembled surface as an artificial secondary surface [217]. It allows the quantitative study of natural state of adhered cells without disturbing cellular physiological activities or requiring major instrument.

Sensitive chemiluminescent imaging method has been developed for in situ monitoring of cell surface glycan expression through chemoselective labeling of carbohydrate motifs and then binding to multifunctional nanoprobes [209]. The nanoprobes can be prepared by assembling biotin-DNA and high amount of HRP on gold nanoparticles, and the chemoselective labeling can be performed by selective oxidization of the hydroxyl sites of sialyl and galactosyl groups on cell surfaces into aldehydes with periodate and galactose oxidase respectively, and then aniline-catalyzed hydrazone ligation with biotin hydrazide for specific recognition to avidin. With the biotin-avidin system, the multifunctional nanoprobe can conveniently be bound to the glycan sites on cell surface to trigger the CL emission of luminal-H_2O_2 system for detecting the expression of both sialyl and galactosyl groups by CL imaging.

Fluorescence-based nanoprobes have been extensively applied in biomedical and clinical diagnostic fields. A functional nanosphere with excellent fluorescence and magnetism was

firstly developed for recognition of cancer cells surface-expressed with sialic acid and N-acetylglucosamine by encapsulation of QDs and nano-γ-Fe_2O_3 in poly(styrene/acrylamide) copolymer and then biofunctionalization with WGA [218]. The same group then used different types of lectins, WGA, PNA and DBA, to functionalize fluorescent-magnetic nanospheres [219]. These lectin-modified trifunctional nanoprobes not only could quantify the different glycoconjugates on A549 cell surface, but also could recognize and isolate A549 cells. To achieve the direct quantification and avoid the interference of autofluorescence, Xu et al. [220] linked mercaptopropionic acid-capped CdTe QDs to Con A for the fabrication of mannose-specific nanoprobes, which could bind to cells to decrease the fluorescence intensity of the nanoprobe solution for detection of cell surface mannosyl groups. This group then used APBA to functionalize QDs for specifically recognizing cell surface sialic acid, which was further amplified by binding polysialic acid-stabilized gold nanoparticles and then the APBA-QDs to increase the amount of bound QDs. After the bound QDs were dissolved to release the Cd^{2+} for sensitizing the fluorescence of Rhod-5N, a method was developed for dynamic monitoring of sialic acid expression variation on cell surface [221].

Nanoprobe based fluorescence imaging can provide amplified fluorescent signal for detection and tracing of cellular functional biomolecules. Chen et al. [222] designed a luminescent nanoprobe through electrostatically assembling APBA on gold nanoclusters to develop a density tunable dendrimeric array for in situ tracing of cell surface glycan density, using terminally-expressed sialic acids as the target. Qian et al. [223] prepared a polysialic acid embedded gold nanoprobe and then functionalized the nanoprobe with fluorescent 3-(dansylamino)phenylboronic acid (DAPB) to develop a method for in situ imaging and dynamic monitoring of cell surface sialic acids.

Surface-enhanced Raman scattering (SERS) can provide the complete vibrational information of molecules, thus Raman signal molecule (RSM) coded nanoprobes have been used for multiple detection. Chen et al. [224] prepared a set of Raman barcoding nanoprobes by coding gold nanoparticles with lectins and RSM to couple with a designed micro-competition system for fast detection of multiple glycans on cell surface. The micro-competition system consisted of the nanoprobes, multiple-polysaccharide-coated gold nanostars assembled on silica bubbles, and cells. The designed anisotropic multiple glycan surface could compete with cell surface glycans for binding different types of nanoprobes via lectin-glycan recognition in a one-molecule-two-surface format at a micron scale, and the amounts of nanoprobes selectively bound on the artificial glycan surface could be detected with SERS spectroscopy to accurately reflect the amounts of glycans on cell surface.

Recently, SERS imaging has emerged as an alternative tool for glycan detection because of its advantages of high sensitivity, non-destructive and non-invasive features and fingerprinting capability on chemical structures [225,226]. The sensitivity of these strategies is usually limited by the distance between the reporter and substrate or the absence of interparticle plasmonic coupling effect. To facilitate the improvement of the sensitivity, Song et al. [227] designed an Au nanoflower (AuNF) based probe as a bridge to recognize the target cell surface sialic acids and assemble poly(N-acetylneuraminic acid) modified Au nanoparticles, which formed a single core-multi satellites nanostructure for global imaging of sialic acids on living cells. As a SERS substrate [228], both the coarse surface and dense tips of AuNF provided the increasing surface area and large amounts of hot spots on their surfaces to produce strong SERS for sensitive imaging analysis.

The glycosylation of proteins profoundly affects cellular adhesion or motility, which further reflects the physiological and pathological states of cells. Thus in situ visualization of glycans on specific protein may provide the status and correlation of protein glycosylation with disease state for uncovering their roles in disease development. Several Förster resonance energy transfer (FRET) methods have been developed for imaging of protein-specific glycans by labeling protein and the corresponding glycan with two FRET-achievable fluorescent molecules [229–231]. However, one donor-to-one acceptor FRET mode cannot provide the integral glycan signal on target protein that is generally modified with more than one glycan. Besides, the short FRET distance between donor and acceptor might limit its application in the study of biggish proteins. To provide the exact glycosylation information of target protein, Chen et al. [232] prepared a substrate probe, an aptamer modified 30 or 40-nm Au nanoparticle, and a signal probe, a RSM and dibenzocyclooctyne-amine functionalized 10-nm Au nanoparticle to design a zone-controllable SERS effect by controlling the size of substrate to match the expression zone of protein-specific glycan (Fig. 5), which led to a strong SERS signal for Raman imaging of protein-specific glycans on cell surface. Moreover, the concept of zone control could also be used for in situ measurement of the space distance of glycoproteins on cell surface. They then designed a strategy for information liberation of protein-specific glycosylation via exonuclease III-aided recycling hybridization with glycan and protein probes to achieve homogeneous quantification of cell surface glycan [233]. The protein probe contained matching and spacer DNA sequences and an aptamer specific to target protein, while the glycan probe contained a complementary sequence modified with neighboring fluorescein and quencher, a spacer sequence and a dibenzocyclooctyne-amine end to bind azide-tagged glycan. Upon sequential binding to their targets, the complementary sequences approached enough for hybridization, which led to the cleavage of hybridized glycan probe by exonuclease III and recycling hybridization of protein probe with other adjacent glycan probes to release fluorescein for obtaining the information of protein-specific glycosylation. This protocol was used to in situ quantify EpCAM-specific sialic acid on MCF-7 cell surface and monitor its variation during drug treatment.

Considering the diversity and complexity of the glycoforms, profiling multiple monosaccharides on a given cell surface protein is very significant for revealing complex glycan-regulated signal pathway machinery. To achieve the simultaneous detection of multiplex glycans on specific cell surface protein, Ju's group used upconversion luminescent nanoparticles (UNPs) to construct a site-specific duplexed luminescence resonance energy transfer (D-LRET) system on cell surface using mucin 1 (MUC1) as the model protein [234]. The aptamer modified UNP acted as an energy donor to target the protein, and two fluorescent dye acceptors tagged two kinds of cell surface monosaccharides by a dual metabolic labeling technique. Upon excitation at 980 nm, only the dyes linked to protein-specific glycans could be lit up by the donor via two paralleled energy transfer processes for in situ duplexed imaging of glycoforms on specific protein. This approach provides a versatile platform for profiling protein-specific glycoforms, thus contributing to studying the regulation mechanisms of protein functions by glycosylation.

4.2. *In vivo detection of cellular functional molecules*

4.2.1. *Intracellular microRNAs*

MicroRNAs (miRNAs) are small noncoding RNAs (~22 nucleotides in length) within plants, animals and virus genomes, which play fundamental roles in a wide variety of biological processes including cell differentiation, proliferation, and apoptosis as well as tumorigenesis processes. Therefore, the detection of miRNAs has become a rapidly emerging field for further understanding the biochemical function of miRNAs and exploring useful diagnostic and prognostic markers of diseases [235–238]. To achieve the in

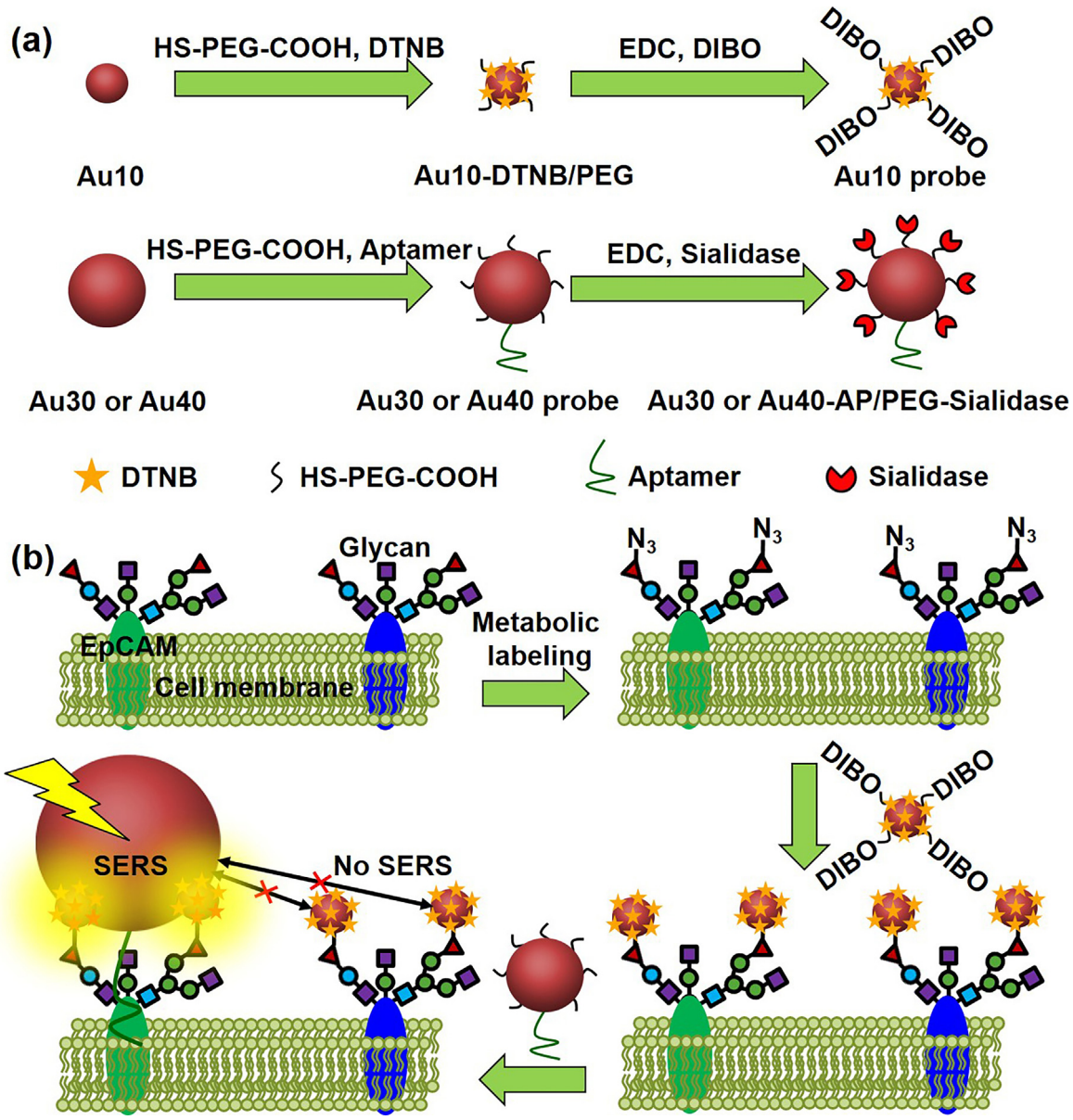

Fig. 5. Schematic illustration of (a) synthesis of two types of Au nanoprobes and (b) zone-controllable SERS effect for imaging of protein-specific glycans on cell surface. Adapted with permission from Ref. [232]. Copyright 2016 The Royal Society of Chemistry.

vivo detection of cellular miRNAs, Dong et al. [239] combined the remarkable affinity and specificity of locked nucleic acid modified on molecular beacon (LNA-m-MB) to miRNA with the large surface area of the graphene nanoribbon (GNR), and the high charge density and the "proton sponge effect" of polyethylenimine for loading of LNA-m-MB of PEI and efficient transfection of cells to prepare a delivery system for effectively transferring the LNA-m-MB functionalized nanoprobes into the cells. The recognition of LNA-m-MB to miRNA opened the MB for fluorescence imaging, which led to the first method to in vivo recognize and in situ detect miRNA in single cell. They then designed a multifunctional SnO_2 nanoprobe (mf-SnO_2) containing folic acid for target-cell-specific cell delivery and LNA-m-MB with pH-sensitive disulfide linkage for recognition of target miRNA to accomplish gene-specific cell delivery with the necessary feature of visualizing the delivery and intracellular response by the fluorescent property of mf-SnO_2 and specifically recognize the target miRNA for in situ detection of the amount of intracellular miRNA [240]. By assembling thiolated RNA to gold nanoparticle and then binding 3′-end amine of the RNA to the carboxy group capped on quantum dot surface, a QD-RNA-Au nanoprobe was prepared, which was immobilized on chiostan/poly(γ-glutamic acid) complex as a gene vector for highly effective cellular uptake and delivery [241]. After the nanoprobe was released from the vector to the cytoplasm by electrostatic repulsion at intracellular pH, it hybridized with pre-miRNA precursor as target, the formed product was cleaved by RNase III Dicer to separated QDs from Au NPs for fluorescence emission of QDs. Thus a method for monitoring the amount of the intracellular pre-miRNA precursor.

The in situ monitoring of intracellular multiple miRNAs can be achieved by assembling different dye-labeled single-strand DNAs (dye-ssDNAs) and then folate acid-Poly A on CNNS through their strong π–π interaction. The loading of dye-ssDNAs on CNNS leads to the quenching of dye fluorescence by CNNS. After the mf-CNNS nanoprobes is cell-target-specifically transfected into the cells, the hybridization of the assembled dye-ssDNAs with complementary targets weakens the π–π interaction between bases and CNNS, which leads to the release of the dye-ssDNAs from CNNS, and thus

recovers the quenched fluorescence for simultaneous detection of multiple miRNAs in a living cell [242]. Similarly, this nanoprobe can be prepared by functionalizing CNNS with Cy5-labeled peptide nucleic acid (Cy5-PNA) and folate acid. Upon the recognition of the PNA to complementary miRNA, the hybridization product is released from CNNS surface to provide a specific method for sensing of miRNA [243].

The fluorescence imaging technique with carbon nanosphere (CNS) as the gene carrier to deliver gene probe into cells has been used for in situ quantitation of intracellular microRNA in whole cell cycle [244]. The nanoprobes can be prepared by co-assembling folic acid and Cy5-labeled ssDNA on CNS surface, and the cells are firstly synchronized at the different phases, respectively. Using miRNA-18a as an analyte model, the calibration curves can be obtained by transfecting miRNA-18a mimic into the cells synchronized at different phases, from which the intracellular miRNA-18a in whole cell cycle can be detected to be 0.748, 0.969 and 1.21 pg in single S-, G2/M- and G1-phase HepG2 cell. The results demonstrated that the proposed cell-target-specific delivery and detection system is attractive for the study of miRNA related cell cycle bioprocesses and clinic biomedical application.

4.2.2. Intracellular enzymatic activity

Glycosylation involves a set of functionally specific glycosyltransferases located mainly in Golgi apparatus. As one class of glycosyltransferases, sialyltransferases (STs) can introduce sialic acid to the terminal position of glycan chain attached on protein. The common methods for ST assay involve the separation of sialic acid from cellular lysate. To achieve the in situ noninvasive evaluation of ST activity, a sensing system was constructed to trace the sialylation process in living cells by encapsulating tetramethylrhodamine isothiocyanate labeled asialofetuin (TRITC-AF) as ST substrate and fluorescein isothiocyanate labeled APBA (FITC-APBA) as the chemoselective recognition probe of sialylation product in a liposome-based delivery vesicle [245]. FITC and TRITC could act as the donor and acceptor of a FRET pair, respectively. After the vesicle was delivered into cells, the intracellular ST could transfer the SA from cytidine-5′-monophospho-sialic acid, which exists in Golgi apparatus, to the terminal position of glycan chains of the TRITC-AF. Upon sialylation of the AF, the product could be recognized by the released FITC-APBA to bring FITC and TRITC close enough to achieve FRET. The signal intensity depended on the formation of the sialylated TRITC-AF, thus could be used for assessment of ST activity.

Neuraminidases (Neus) are a family of glycosidases responsible for removing α-glycosidically linked sialic acid residues from glycoconjugates. They locate at different cellular compartments such as lysosome, cytosol, plasma membrane and mitochondrion, and play a central role in regulating cell-surface sialic acid expression. Currently available protocols for lysosomal neuraminidase (Lyso-Neu) activity assay are limited to cell lysates. Although a cell-permeable probe has been designed to label intracellular Neus via irreversible binding, it fails to reflect the enzymatic cleavage activity [246]. Therefore, Bao et al. [247] proposed a cell-permeable and lysosome-accessing nanoprobe to efficiently deliver Lyso-Neu-specific substrate into lysosomes and release cleavage product into cytosol for neutral pH-enhanced fluorescent detection of Lyso-Neu activity, which led to a light-up imaging strategy for monitoring of Lyso-Neu activity in living cells. The nanoprobes was by assembling 4MUNA on graphene oxide as the “carrier & locater” via a linker, which could be specifically cleaved by Neus in acidic lysosome (pH 5) to produce free 4-methylumbelliferone (4MU) moiety with strong fluorescence at neutral pH. To facilitate the escape of the cleaved 4MU into neutral cytosol for producing strong signal, a cationic polymer, branched poly(ethyleneimine), was co-assembled on graphene oxide surface, which caused lysosomal osmotic swelling to form holes for escape of 4MU into the cytosol due to the unique proton-sponge effect. The proposed light-up imaging strategy provided a powerful tool to track the dynamic variation of Lyso-Neu activity.

Telomerase is a ribonucleoprotein reverse transcriptase to add repeated DNA sequence TTAGGG to the 3′ end of telomeres in cells. The analysis, particularly in situ monitoring, of telomerase activity, is significant to cancer diagnosis, therapy and monitoring. Different methods have been developed for probing the telomerase activity, but they fail to in situ detect and provide telomerase information at single-cell level. To provide in situ detection protocol for monitoring intracellular telomerase activity, Qian et al. [248] used MSN to design a telomerase-responsive probe by entrapping fluorescein in the mesopores of MSN with a specially designed wrapping DNA (O1) and covalently immobilizing black hole fluorescence quencher (BHQ) on the inner walls of the mesopores. The O1 contained repeated CCCTAA and TP sequence and could act as a “bio-gate”. The in situ synthesis of telomeric repeats at the 3′ end of O1 led to the detachment of O1 from the probe surface to trigger the release of fluorescein, thus turned “on” its fluorescence. This switchable probe provided a powerful protocol for “off-on” fluorescent imaging of intracellular telomerase activity. They then designed a smart vesicle kit by co-encapsulating Cy5-tagged molecular beacon functionalized gold nanoparticles and telomerase primer (TSP) in liposome [249]. In the presence of telomerase and dNTPs, the 3′ end of TSP could be elongated to generate telomere repeat sequences complementary with the loop of MB, thus open the hairpin and release the Cy5 away from the surface of AuNPs, which turned “on” the fluorescence for in situ quantification and dynamic monitoring of intracellular telomerase activity. Considering the complexity in the preparation of nanoprobes and their stability, and the multiple stages involved in response process, this group designed a nicked molecular beacon to functionalize Au nanoparticles [250]. The nick separates the molecular beacon into two segments: the shorter telomerase primer (TSP) sequence as a part of 5′-end stem, and the longer sequence for forming the loop with one thiol-labeled 3′-end stem. Upon endocytosis of the probe, the nicked molecular beacon could be opened by substitutional hybridization of telomerase-triggered TSP stem elongation product to produce telomerase activity-related fluorescence signal (Fig. 6). Thus this probe could be conveniently used for in situ imaging and detection of intracellular telomerase activity through a one-step incubation procedure. The practicality of this approach for distinguishing tumor from normal cells and monitoring the decrease of telomerase activity during treatment with anti-tumor drug demonstrated its potential in clinical diagnostic and therapeutic monitoring. To improve the sensitivity of in situ telomerase detection method, a cascade amplification approach was developed for visualization of telomerase activity in living cells by linking telomerase-responsive primer extension with catalyzed hairpin assembly-based signal amplification in series through a DNA transmitter, which achieved multiplied enhancement of signal outputs from one extension event due to the recycling use of the transmitter [251]. The proposed strategy relied on the synthesis of a dual function module-encapsulated liposome nanoprobes. The first module was a telomerase-targeting responder-transmitter DNA complex, and another contained a pair of metastable DNA hairpin (H1, H2), and a hybridized reporter complex (R-FQ) with strands respectively labeled by a fluorophore (F, FAM in this work) and quencher (Q, Dabcyl in this work). To ensure the sensitive and selective long-term intracellular tracing, locked nucleic acid nucleotides were also incorporated into the reporter complex, owing to their excellent thermal stability and enzymatic resistance capability in the cell cytoplasm.

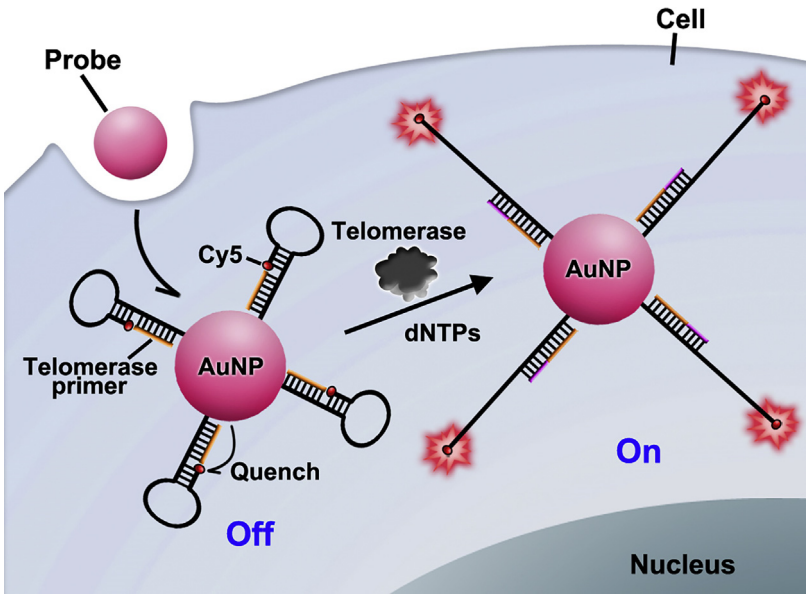

Fig. 6. Schematic illustration of the nicked molecular beacon-functionalized gold nanoparticle (Probe) for in situ analysis of intracellular telomerase. Adapted with permission from Ref. [250]. Copyright 2014 American Chemical Society.

4.2.3. Intracellular ATP

ATP is an essential biogenic biomolecule and plays important roles in a variety of biological processes. The concentration of intracellular ATP ranging from 1 to 10 mM is much greater than that in body fluid (<5 μM). The distinct difference of the ATP levels provides a significant principle for designing ATP-mediated drug delivery platforms and the related in vivo monitoring method. This detection principle can be achieved by simply assembling chlorine e6 (Ce6) labeled ATP aptamer on MoS_2 nanoplates [252]. After the nanoprobe is internalized into the cells and entered ATP-abundant lysosomes, its recognition to ATP leads to the release of the single-stranded aptamer from MoS2 nanoplates and thus recovers the quenched fluorescence of Ce6 at an excitation wavelength of 633 nm, which produced a highly sensitive and selective method for imaging of intracellular ATP. Meanwhile, the ATP-mediated release leads to the generation of $^1O_2$ under 660-nm laser irradiation, which can induce tumor cell death with a lysosomal pathway. The controllable photodynamic therapy (PDT) provides a model approach for design of multifunctional theranostic nanoprobes and can also promotes the application of MoS_2 nanoplate-based platforms in biomedicine.

4.2.4. Intracellular caspases

Caspases are a family of cysteine-aspartic proteases that are only activated during cell apoptosis, and have been used as feedback markers of cell death and the sensors for evaluating therapeutic responses against cancer cells [253,254]. The activation imaging of caspases can sense the cell apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligands, doxorubicin and staurosporine [255]. Moreover, caspase-controlled apoptosis has a characteristic enzyme cascade, which involves multiple caspases at different stages and pathways. For example, upstream caspase, such as caspase-9 (casp-9), plays a central role in the induction of apoptosis, while downstream caspase such as caspase-3 (casp-3) is critical for carrying out the final step of cell apoptosis. Thus the evaluation of intracellular caspase family is essential to elucidate the cell apoptosis process. Many fluorescent probes have been developed for imaging of caspase activity in living cells and animals [256,257], and real-time monitoring of caspase cascade activation by diverse pairs of dyes and corresponding quenchers [258]. These methods usually need additional inducers to activate intracellular caspase activity [259]. After the cells are incubated with the nanoprobes responsive to caspase, they are then treated with apoptosis inducer at different concentrations to trigger the change of caspase activity. The apoptosis sensor for in situ activation and imaging of intracellular casp-3 using aggregation-induced emission has been proposed to avoid the need of inducers [254]. Considering the apoptosis-inducing capability of porphyrin derivatives and the structural diversity and tenability of MOFs. A multifunctional nanoprobe has been designed by integrating the porphyrin derivatives and MOFs in single nanoprobe for highly efficient therapy and monitoring of the therapeutic effectiveness via caspase-dependent apoptosis imaging [260]. The porphyrin derivative, tetrakis(1-methylpyridinium-4-yl)porphyrin (TMPyP), as photosensitizer, is incorporated in the cage of a variant MOF by one-pot synthesis to form PS@MOF. A Cy3-labeled caspase-3 substrate peptide along with H_2N-PEG-folate (FA) as a target specific moiety is covalently assembled on PS@MOF surface. The MOF can significantly increase the singlet oxygen ($^1O_2$) quantum yield of TMPyP by 6.5 times under a 635-nm irradiation, and quench the fluorescence of Cy3. Upon endocytosis of the nanoprobe and laser irradiation, cell apoptosis is encouraged by the therapeutic effect of probe-mediated $^1O_2$, and thus activates caspase-3 to specifically cleave the peptide on the probe, which releases the Cy3 from MOF surface for fluorescent imaging and caspase-responsive monitoring of cell apoptosis. The turn-on signal provides an efficient way to image intracellular caspase activity for the real-time evaluation of therapeutic effectiveness. This strategy can be further developed to in situ activate and monitor the evolution of caspase family during cell apoptosis. Using gold nanorod (AuNR) as the model of both nanocarrier and matter inducing the cell apoptosis, which has been found to be able to simultaneously quench two kinds of dyes at two unique surface plasmon resonance (SPR) absorption wavelengths, a versatile nanoprobe has been designed for in situ activating and monitoring the evolution of caspase family from upstream to downstream via NIR photothermal treatment [261]. The nanoprobe was prepared by assembling FITC-labeled peptide specific to casp-9 (peptide-9) and cyanine-5.5 (Cy5.5)-labeled peptide specific to casp-3 (peptide-3) as signal switches and recognition elements, and folic acid (FA) as a target specific moiety on AuNR (Fig. 7). Their fluorescence was initially quenched via energy transfer from FITC and Cy5.5 to AuNR with transverse and longitudinal SPR absorption,

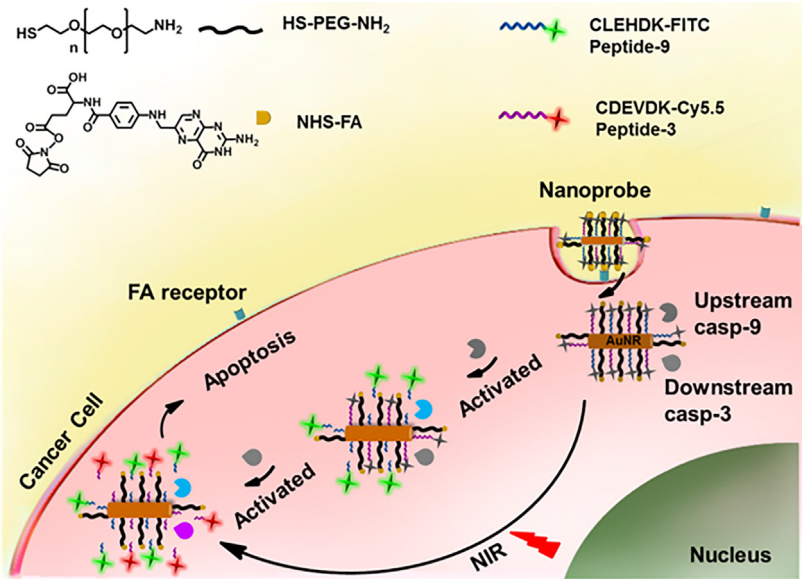

Fig. 7. Schematic illustration of an integrated platform for in situ monitoring the evolution of caspase family activated via real-time NIR photothermal therapy. Adapted with permission from Ref. [261]. Copyright 2016 The Royal Society of Chemistry.

respectively. Upon endocytosis of the nanoprobe and NIR irradiation, cell apoptosis was encouraged by the photothermal effect and thus the peptide could be cleaved successively by corresponding activated caspase from upstream casp-9 to downstream casp-3, which released the dyes from the nanocarrier for fluorescent imaging. The turn-on signals provided an efficient way for quantification of both caspa-9 and casp-3 activities in cancer cells and monitoring of their evolution in living mice. The average activity of casp-9 and casp-3 in a single HeLa was cell was 4.25 and 5.09×10^{-7} Unit after the therapy-inducing apoptosis. After tumor-bearing mice were intravenously injected with nanoprobes foe 24 h, and irradiated with a NIR laser for 30 min to perform the therapy, tumor volume reduced gradually, and the fluorescence intensity for Cy5.5 and FITC from the irradiated tumor increased. Furthermore, the fluorescence of FITC for casp-9 showed earlier and greater change than that of Cy5.5 for casp-3, indicating that casp-9 was activated prior to casp-3. Since caspase activity is the marker of cell apoptosis, the fluorescence response could also be used to monitor the therapeutic efficacy in real time.

5. Conclusions and outlook

The usage of nanomaterials has been widely spread over the last twenty years in the development of conceptually new biosensors [262]. Although tremendous advances have been achieved in the exploring of nanomaterial-based signal amplification strategies for the development of ultrasensitive biosensing methods, and the emergency bioconjugated nanomaterials have demonstrated their broad potentials in amplified transduction of recognition events, the variability of the preparation of nanomaterials and their biofunctionalization often affect the reproducibility and quantification of these biosensing methods, especially for the real samples. The detection of genes and specific proteins at ultra-low levels in real sample is still particularly challenging. Thus, extensive effort is still an urgent demand to improve the reproducibility and practicability of the nanomaterial-based signal amplification strategies. The control of synthetic methods for nanoparticles or nanoprobes will be an efficient way to improve the analytical performance [263,264]. The integration of these strategies with different DNA-based signal amplification techniques such as RCA, HCR, catalyzed hairpin assembly and DNA-fueled molecular machine have significantly improved the sensitivity of bioanalysis [265], which will ultimately dissolve the bottleneck problem in detection of biomolecule with low abundance and acquisition of ultraweak biological signals. The exploration of new signal amplification techniques with high throughput and high sensitivity are also an urgent demand for the validation and routine application of biomarkers.

Congratulations

Dear Joe,
Congratulations and best wishes on your 70th birthday. As your collaborator, I wish to extend my special congratulations to you, a leader in the development of many chemical and biological sensors. You are a cheerful, hard-working and extremely successful scientist. Your important works on behalf of Electroanalysis and Sensors have been benefited many people a great deal, both within and outside academia. You will probably never know how many people like me deeply appreciate you and celebrate your creativity and wisdom.
I wish your health be great. Happy Birthday to you, Joe!
Huangxian

Acknowledgments

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